



Short communication

Quantification of pyrophosphate in soil solution by pyrophosphatase hydrolysis

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ABSTRACT

A commercial pyrophosphatase from *Saccharomyces cerevisiae* selectively hydrolyzed sodium pyrophosphate, but showed no significant activity towards a range of other organic and condensed inorganic phosphorus compounds. Pyrophosphate determined by pyrophosphatase hydrolysis accounted for $38 \pm 12\%$ (mean \pm standard error of 19 sites) of the non-reactive phosphorus in soil solution obtained by centrifugation from a series of lowland tropical rain forest soils. Pyrophosphate concentrations were up to $89 \mu\text{g P l}^{-1}$ and correlated positively with microbial phosphorus, soil solution pH, and native phosphomonoesterase activity in soil solution, but not with total soil pyrophosphate determined by NaOH–EDTA extraction and solution ^{31}P NMR spectroscopy. In summary, we identify pyrophosphate as a major constituent of soil solution phosphorus in lowland tropical rain forests, and demonstrate that a commercial pyrophosphatase can be used as a selective tool to quantify trace concentrations of pyrophosphate in soil solution.

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Pyrophosphate is ubiquitous in soils, where it appears to originate mainly from soil fungi (Rasmussen et al., 2000; Makarov et al., 2005; Bünemann et al., 2008). However, the role of pyrophosphate in the nutrition of plants remains poorly understood, due in part to the difficulty in its quantification at low concentrations in soil solution. Ion chromatography was used to assess the fate of pyrophosphate fertilizer added to soils (e.g., McBeath et al., 2007), while pyrophosphate can be quantified in alkaline soil extracts by solution ^{31}P NMR spectroscopy (e.g., Turner et al., 2003). However, the latter procedure is inappropriate for analysis of soil solution due to its relative insensitivity.

Several studies have used phosphatase enzymes to detect phosphorus compounds in soil solution or soil water extracts (e.g. Shand and Smith, 1997; Hayes et al., 2000; Turner et al., 2002). Phosphatases added to soil solution or extracts catalyze the hydrolysis of specific functional organic phosphorus groups, allowing detection of the released orthophosphate by routine colorimetry. Phosphatase hydrolysis procedures are sufficiently sensitive to quantify trace concentrations of organic phosphates in solution, but established protocols have not identified pyrophosphate separately from simple phosphomonoesters (reviewed in

Bünemann, 2008). As a result, data on soil solution pyrophosphate remains scarce.

We tested the specificity of a commercially available pyrophosphatase from *Saccharomyces cerevisiae* (baker's yeast; Sigma product number 9024-82-2) towards a range of model phosphorus compounds and then applied this technique to soil solution obtained by centrifugation from a series of lowland tropical rainforest soils in the Republic of Panama. Soil solution is of interest because it is the source of nutrients for plant uptake. Pyrophosphatase was tested against 14 different organic phosphorus compounds and three condensed inorganic phosphates. All assays were conducted in 96 well microplates, with each well receiving 152 μl of sample, 28 μl of 2-morpholinoethanesulfonic acid (MES) buffer (pH 5.5), and 20 μl of pyrophosphatase prepared in buffer. Although the optimum pH of the enzyme is 7.0, we used a pH 5.5 buffer to approximate the mean pH of soil solution in our study sites to minimize any changes in soil solution chemistry that might influence the assays. The MES buffer contained 2 mM magnesium chloride to stimulate pyrophosphatase activity and 1 mM sodium azide to eliminate microbial activity during the assay without lysing microbial cells (Turner et al., 2002). The final buffer concentration in each well was 15 mM. Solutions of authentic phosphorus compounds ($200 \mu\text{g P l}^{-1}$) were incubated at 35 °C with pyrophosphatase (final activity 0.1 nkat ml^{-1}) for 24 h and the soluble reactive phosphate (SRP) concentration determined

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colorimetrically by the malachite green method (Irving and McLaughlin, 1990) with a detection limit of $6 \mu\text{g P l}^{-1}$ (D'Angelo et al., 2001). The difference between SRP in control and enzyme-treated solutions was assumed to represent pyrophosphatase-hydrolyzable phosphorus. Samples containing buffer solution, pyrophosphatase and commercial pyrophosphate were always included in the soil solution assays to verify that the enzyme activity was sufficient to completely hydrolyze $200 \mu\text{g P l}^{-1}$ of pyrophosphate within 24 h.

The pyrophosphatase was remarkably specific to the target substrate, hydrolyzing 100% of sodium pyrophosphate, but $\leq 2\%$ of a series of phosphomonoesters, phosphodiester, organic polyphosphates, and phosphonates. Pyrophosphatase released no phosphate from an authentic sample of long-chain polyphosphate ($n = 45$), although we recommend that enzyme specificity should be tested against additional chain types in future studies.

To assess the applicability of the pyrophosphatase to environmental samples, we collected soil solution from a series of 19 sites under lowland tropical rainforest in the Republic of Panama (see Turner and Engelbrecht, 2011 for details of soil properties of the majority of the sites). At each site, four replicate soil samples were collected during the 2012 wet season. Soil solution was isolated by centrifugation of 1.2 kg soil for 15 min at $10,000 \text{g}$ (Giesler et al., 1996) and then filtered through a $0.45 \mu\text{m}$ cellulose-acetate membrane (Whatman) on the day of collection. Within 24 h we determined SRP and pyrophosphatase-hydrolyzable phosphorus as described above. We also determined total dissolved phosphorus (TDP) by persulfate oxidation (Koroleff, 1983) and calculated non-reactive phosphorus as the difference between TDP and SRP. Microbial phosphorus was measured as phosphorus released following hexanol fumigation (i.e. without application of a correction factor; Kouno et al., 1995). Native phosphomonoesterase activity in the soil solution was measured by fluorescent substrates (Turner and Romero, 2010). Whole-soil pyrophosphate was determined by NaOH–EDTA extraction and solution ^{31}P NMR spectroscopy (Turner and Engelbrecht, 2011).

Pyrophosphatase-hydrolyzable phosphorus (i.e. pyrophosphate) concentrations were on average $31 \pm 6 \mu\text{g P l}^{-1}$ (mean \pm standard error of 19 sites) and accounted for $38 \pm 12\%$ of the non-reactive phosphorus in soil solution. Mean concentrations for individual sites were up to $89 \mu\text{g P l}^{-1}$ (Fig. 1) and accounted for up to 100% of the non-reactive phosphorus. Pyrophosphate concentrations were correlated positively with microbial phosphorus, native phosphomonoesterase activity in the soil solution, and pH of the soil solution (Fig. 1). However, there was no significant correlation ($p > 0.05$) between soil solution pyrophosphate measured by enzyme hydrolysis and whole-soil pyrophosphate determined by NaOH–EDTA extraction and solution ^{31}P NMR spectroscopy (Fig. 1). A simple estimate based on site averages indicates that pyrophosphate in soil solution constituted on average $<1\%$ of the pyrophosphate determined by NMR spectroscopy (assuming 30% water content and a bulk density of 1.0g cm^{-3}).

The strong correlations between pyrophosphate in soil solution, native enzyme activity, and microbial phosphorus suggest that soil solution pyrophosphate has a microbial origin. Indeed, several studies have reported high concentrations of pyro- and polyphosphate in soil fungal tissue (Makarov et al., 2005; Bünemann et al., 2008; Koukol et al., 2008). However, the absence of a correlation between soil solution pyrophosphate and pyrophosphate determined by ^{31}P NMR spectroscopy suggests that these pools are decoupled, perhaps because ^{31}P NMR spectroscopy detects a relatively stable pyrophosphate pool that is sorbed strongly to the mineral soil matrix (Gunary, 1966) or contained within live fungal tissue (Koukol et al., 2008; Cheesman et al., 2012).

These results demonstrate that pyrophosphate constitutes a quantitatively important, but poorly understood, form of

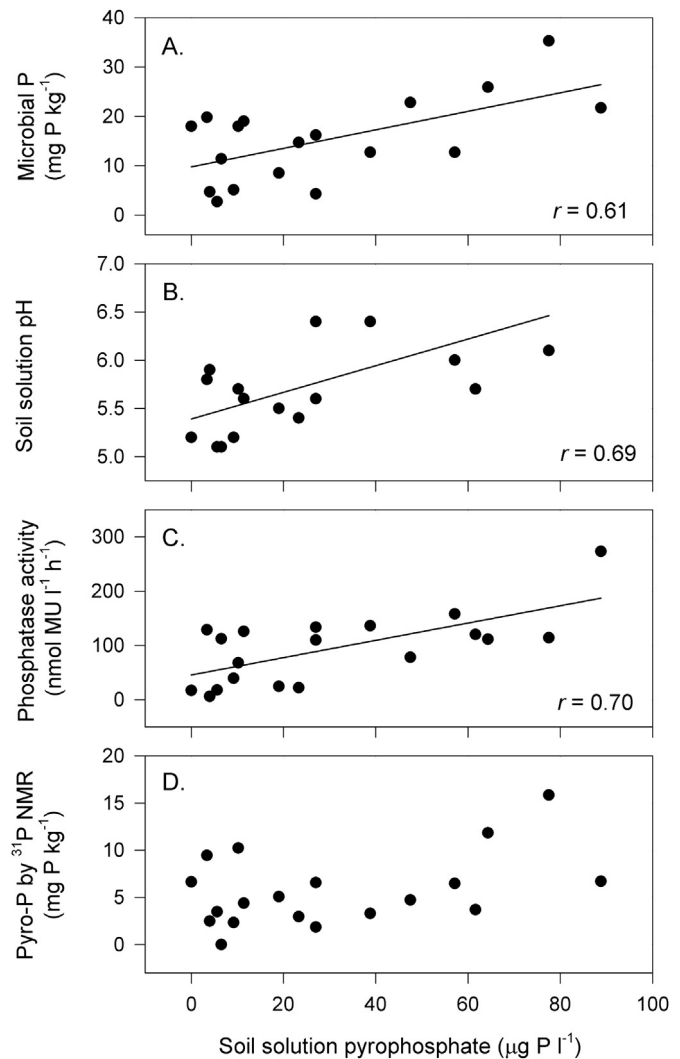


Fig. 1. Correlations between soil solution pyrophosphate and (A) microbial phosphorus, (B) pH in soil solution, (C) native phosphomonoesterase activity in soil solution, and (D) whole-soil pyrophosphate determined by NaOH–EDTA extraction and solution ^{31}P NMR spectroscopy. Pearson's correlation coefficients (r) are shown and are significant at $p < 0.008$. Data on pyrophosphate determined by NMR spectroscopy were non-normally distributed and were \ln transformed for analysis. In the Y-axis units in (C), MU is the hydrolysis product methylumbelliferone.

biologically available phosphorus in the soil solution of lowland tropical rainforests. This agrees with previous reports that dissolved condensed inorganic phosphate (i.e. pyro- and polyphosphates) constituted a considerable proportion of the TDP in soil solutions from UK grasslands (e.g., Ron Vaz et al., 1993; Shand et al., 2000). The labile nature of soil solution pyrophosphate (McBeath et al., 2008) supports the hypothesis that transpiration-induced mass flow can supply a considerable amount of hydrolyzable phosphorus compounds to the root surface and therefore contribute to phosphorus nutrition of the forest (Cernusak et al., 2011).

In addition to providing new insight to the role of pyrophosphate in the environment, the use of pyrophosphatase in combination with other enzymes will allow a more precise identification of phosphorus fractions by enzyme-hydrolyzable phosphorus procedures in soil and waters. The pyrophosphatase assay offers a highly substrate-specific and sensitive method to identify and quantify pyrophosphate in soil solution, and should be considered in future enzyme-hydrolyzable phosphorus assays.

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